Characterization of probiotic cultures from fermented foods of Himachal Pradesh and their efficacy against colonization by *Cronobacter sakazakii* in *Caenorhabditis elegans* model system

SYNOPSIS

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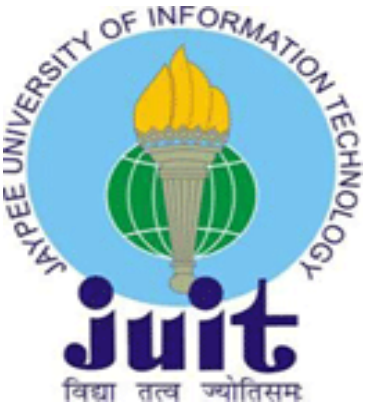
BY

KAVITA SHARMA



DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT-173234, HP, INDIA

Introduction

Traditional fermented food products have been reviewed in terms of their traditional processing technology and the significant role of lactic acid bacteria (LAB) in enhancing their organoleptic, preservative, and nutritive value [1]. Himachal Pradesh, is a state in the lap of Himalayas which is highly diverse in its topography, culture and has a long history of numerous fermented food products, produced largely in household scale or small scale units [2]. Most of these fermented dairy or cereal based products are artisanal and are closely related to the indigenous microbiota, which makes them a pertinent source of beneficial indigenous LAB as starters [3].

Probiotics are non-pathogenic microorganisms, which when administered into the body in adequate amount exert a positive health benefit on host [4]. Different probiotic bacterial genera with known health benefits include *Lactobacillus, Bifidobacterium, Saccharomyces, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Bacillus, Escherichia coli* and are generally regarded as safe [5]. Among these genera, LAB are involved in the fermentation and are dominant microflora of fermented food products. They are known to play an essential role in food preservation and inhibit spoilage microorganisms or food borne pathogens by production of various components like organic acids, H_2O_2, bacteriocin, diacetyl and CO_2 [6]. Consumption of these LAB probiotics have been shown to be helpful in overcoming various clinical conditions ranging from infectious illness (diarrhoea, uro-genital infections and others), immune system modulation and increased resistance to malignancy [7], slowing the growth of colon cancer, lowering cholesterol levels, alleviating constipation and treating food allergies and also helps in preventing antibiotic associated diarrhoea by acting on the immune system [8].However, to confer the health benefits, the bacterial cultures should pass the challenge to Resistance to low pH, bile acid resistance and Bile salt hydrolase activity, antimicrobial activity against potentially pathogenic bacteria and adhesion ability to reduce pathogen adhesion to surfaces as per ICMR-DBT guidelines for evaluation of probiotics in food.

Once the probiotics are screened through *in vitro* tests, the ability of probiotics to adhere to intestinal epithelial surface and colonize the gastrointestinal (GI) tract are further two of the most important selection criteria [9-11]. Most of the studies have reported the screening of LAB for these parameters using *in vitro* models such as adherence to human intestinal cells (HT-29 and Caco-2 cells), and mucin-adhesion assays[12, 13]. To increase selection efficiency of a probiotic

candidate, *Caenorhabditis elegans*, a free living bacterivorous soil nematode, has been suggested as an *in vivo* model. This *in vivo* model is one step closer to a multicellular animal system with intact intestinal microbiota [14]. The other advantages of using *C. elegans* include its transparent body, which allows clear observation of all cells at all developmental stages [15] and the intestinal cells are similar in structure to those in humans [16].*C. elegans* has been successfully used as established model to study antioxidative, anti-infective and lifespan extending impact of lactobacilli [17].

C. sakazakii is an opportunistic pathogen and an occasional contaminant in powdered infant formula, as infant food made of powdered infant formula products may be a source and vehicle of infections causing meningitis, bacteremia and necrotizing enterocolitis in neonates with case fatality rates ranging between 40% and 80% [18, 19]. Intermediate sensitivity has been described by Cronobacter spp. When bacterial cells were dried onto a solid surface in the presence of an organic matrix the efficacy of the disinfectant was reduced. Populations of cells were not significantly reduced when present as part of a biofilm. Bacteria living in biofilms are physiological very distinct from their planktonic counterparts, and they function as a cooperative consortium more similar to that of multicellular organisms than a unicellular organism [20]. To overcome this, biocontrol is one possible measure that could ensure foods and surfaces are free from contamination.

Rationale

The population of Himalayan region is known to consume variety of fermented food products and as a result have benefited in terms of overall health, because of the associated beneficial microbes. Moreover, the fermented food products could serve as delivery vehicle for probiotics as these foods are already rich with diverse species of LAB. Therefore, the present study is focused on bioprospecting novel strains of LAB from different fermented and non–fermented dairy/ cereal based products and their evaluation for probiotic functional attributes.

C. sakazakii is an emerging food borne pathogen, causing life-threatening forms of neonatal meningitis, sepsis, and necrotizing enterocolitis (NEC) in newborn and premature infants, with a fatality rate of 40% to 80%. The environmental source of *C. sakazakii* is not evidently understood however, the epidemiological studies implicate reconstituted powdered infant formula (PIF) as the possible source of transmission. Our laboratory has previously reported the

prevalence of *C. sakazakii* among different food and environmental commodities. The isolates expressed different virulent factors and were reported to form biofilm mediated by expression of acyl homoserine lactones (AHLs) as signaling molecules and genes encoding for cellulose synthesis. Although probiotic cultures have been reported for their antimicrobial activities against an array of pathogens leading the gastrointestinal disorders, however very few studies have been conducted to check the efficacy of probiotics against *C. sakazakii*. Therefore, the present study was designed to isolate and characterize probiotic cultures from traditional foods of Himachal Pradesh and their efficacy against colonization by *Cronobacter sakazakii* in *Caenorhabditis elegans* model system.

Objectives

1) To isolate and identify bacterial cultures from traditional fermented foods of Himachal Pradesh.
2) To screen the bacterial isolates for probiotic attributes using i*n vitro*methods.
3) To study the effect of selected isolates for probiotic properties using model system *C. elegans*.
4) To study the protective effect of probiotics against infection with *C. sakazakii* in *C. elegans*

Objective 1: To isolate and identify bacterial cultures from traditional fermented foods of Himachal Pradesh.

LAB from different fermented foods and beverages are highly valued for their probiotic properties. Spontaneously fermented foods may constitute a reservoir for new LAB strains with potential probiotic characteristics. Traditionally fermented products have been considered as the best carrier for probiotics. A large number of potentially probiotic microorganisms are present in different kinds of food products which are still unknown and uncharacterized (Ramosa et al. 2013, Sonar and Halami, 2014). Studies regarding bio-prospecting of LAB and their associated functional probiotic attributes involved in production of these products are limited [21]. In northwest (NW) Himalayas, the people prepare and consume a number of traditional foods and beverages. An attempt has been made for the characterization of LAB with probiotic potentials from the traditional fermented foods and beverages of different regions of Himachal Pradesh. The autochtonous nature of the food fermenting microbiota of origin, as well as the GRAS status of LAB species, allows to employ these strains as starter cultures in food fermentations, with the added value of providing health promoting traits. The present study is focused on exploration and characterization of indigenous fermented foods and dairy products from north-west Himalayas for the presence of Lactobacilli and studies the probiotic potential of selected isolates.

Methodology

A total of 150 samples containing fermented dairy products (100 samples) and fermented cereal products (50 samples) were collected from different regions of Himachal Pradesh. The samples were further processed using serial dilution method for the isolation of microbial cultures on MRS (de Mann Rogosa Sharpe) (Hi-media Labs, Mumbai) agar. The plates were incubated at at 37ºC for 48 h. The dominant and distinct isolated colonies were selected from MRS agar plates and sub-cultured to ensure their purity and maintained on MRS agar slants. All the results were compared with standard probiotic strain *Lactobacillus rhamnosus* GG which was procured from NDRI, Karnal. Preliminary identification was done using Gram's stain reaction and observing cell morphologyand catalase test as per standard microbiological protocols. Biochemical identification was done by sugar fermentation test. For safety attributes, hemolytic activity and

antibiotic susceptibility were conducted. The selected isolates were identified by molecular approaches targeting 16S ribosomal RNA gene sequencing [22].

Results and Discussion:

A total of 101 morphologically distinct colonies (creamish/white and opaque) were obtained on MRS agar from 150 different samples. As a preliminary step for identification of potential functional culture, subtractive screening based on hemolytic activity was used resulting in 41 non-hemolytic strains. All the non-hemoltyic isolates were tested for their potential to tolerate gastric pH and intestinal fluid, from which only 15 isolates were able to tolerate simulated gastric conditions and used in the present study for other functional attributes.

All the 15 isolates showed different patterns for utilization of different sugars. Based on the color change in the indicator by the LAB fermentation of respective sugar, the carbohydrate fermentation results in either the species identification (noted as good or doubtful) or inconclusive results. Most of the isolates were not able to utilize mellibiose, raffinose, arabinose and trehalose whereas majority of the cultures were able to ferment galactose, maltose and mannose.Isolate *L. fermentum* K75 was not able to ferment most of the sugars expect Maltose and Sucrose.

In the antibiotic susceptibility test the lactic isolate HM29 (identified as *Brevibacillus thermoruber*) was found to be resistant to all the tested antibiotics (Table 1). The other isolates possessed different levels of susceptibility to the antibiotics. Most of the isolates were found resistant to penicillin, bacitracin, vancomycin, kenamycin and clindamycin and sensitive to amoxyclav, carbemicillin. The isolates were found to be susceptible to ampicillin and resistant to gentamycin except standard strain. Most of the isolates were resistant to vancomycin. A retrospective review on pathogenic relevance of *Lactobacillus*-associated infections reported that the LAB isolates were also mostly susceptible to antibiotics such as erythromycin andclindamycin, except the species-dependent intrinsic resistance to vancomycin which held true for susceptibility towards erythromycin and resistant to vancomycin.

The most promising isolates with potential probiotic attributes were identified using 16S rRNA gene sequencing.

Table 1: Accession numbers of isolates identified by 16S rRNA gene sequencing

Isolates	Identified isolates	Accession number
CD4	*L. paracasei* CD4	KX398050.1
BTM7	*L. gastricus* BTM7	KX398051.1
BTM9	*Brevi. aydinogluensis* BTM9	KX583600.1
CD13	*Brevi. thermoruber* CD13	KX583601.1
GTM14	*Enterococcus sp* GTM14	KX583602.1
HM29	*Brevi. thermoruber* HM29	KX583603.1
HM34	*Brevi. thermoruber* HM34	KX583604.1
CD1	*W. confuse* CD1	KX583605.1
K75	*L. . fermentum* K75	MF927597.1
K78	*L. . fermentum* K78	MF927688.1
K84	*L. . plantarum* K84	MF455228.1
K90	*L. . plantarum* K90	MF455229.1
K94	*P. acidilactici* K94	MF455252.1
K98	*P. acidilactici* K98	MF457590.1
K100	*L. . fermentum* K100	MF944224.1

Conclusion

Fifteen different isolates were obtained from different food commodities. The dairy and cereal based fermented foods are reservoir of probiotic cultures. Molecular identification has confirmed that most of the isolates belong to the group of lactic acid bacteria.

Objective 2: To screen the bacterial isolates for probiotic attributes using i*n vitro* methods

The intestinal microbiota plays an important role in the health of the host because it is involved in nutritional, immunologic, and physiological functions. The intestinal microbiota could influence host of health (O'Sullivan et al., 2005). Probiotics must survive in the acidic gastric environment and colonize the gastrointestinal track. Probiotics are known to be beneficial not only for adjusting intestinal balance but also for their anti-inflammatory, antioxidant, and anticancer effects. However, individual strains have to be tested for each property because the characteristic ascribed to a probiotic are strain-specific. The protective effects of probiotic bacteria against gastrointestinal pathogens have been studied using various antimicrobial mechanistic techniques such as the production of organic acids and other antimicrobials or inhibition of adhesion of pathogens in *in vitro* cell culture (Lee, Kim, Han, Eom, & Paik, 2014; Monteagudo-Mera et al., 2012). Several studies have shown that ingestion of probiotics alleviates the pathological condition of oxidative stress-related model diseases, implying that probiotics can serve as antioxidants to animals. Therefore, the isolates obtained in the first objective were further screened for their activity towards different probiotic attributes as outlined in guidelines of DBT-ICMR, Govt. of India.

Methodology: All the fourteen isolates screened in first objective were subjected to the following tests for probiotic attributes:

Screening of lactic acid bacteria for probiotic attributes

- Tolerance to Low pH, Bile Salts, and Simulated Gastric and Intestinal Fluids [23].

Adherence potential of lactic cultures

- Cellular autoaggregation[24].
- Cell surface hydrophobicity [25].
- Bacterial adhesion to Caco 2 cell lines [26].
- Adhesion capacity to intestinal mucus.

Functional properties

- Antioxidant activity (DPPH and ABTS) [27].

- Bile salt Hydrolase activity [28].
- Cholesterol-lowering assay [28].
- Exopolysaccharide production

Results and Discussion:

All the isolates were able to tolerate low pH 2.0 of gastric phase, except *Brevibacillus aydinogluensis* BTM9 and *Brevibacillus thermoruber* HM29. All the isolates under study were tolerant to bile concentration of 1% and pancreatin at a concentration of 1mg/ml. All the isolates were positive for bile salt hydrolase activity as depicted by white precipitation around the colonies on bile supplemented agar plates. The 'bsh' activity was further confirmed by obtaining an amplicon of 250 bp against bsh primers tested. The tolerance of the LAB strains to simulated gut conditions at low pH and bile salt conditions revealed nearly one log decrease in the CFU count after one hour of incubation at pH 2.0 ($p<0.05$), however at pH 3.0, no decrease in count was observed which might be due to adaptation of isolates to acid at the time of their presence in MRS broth.

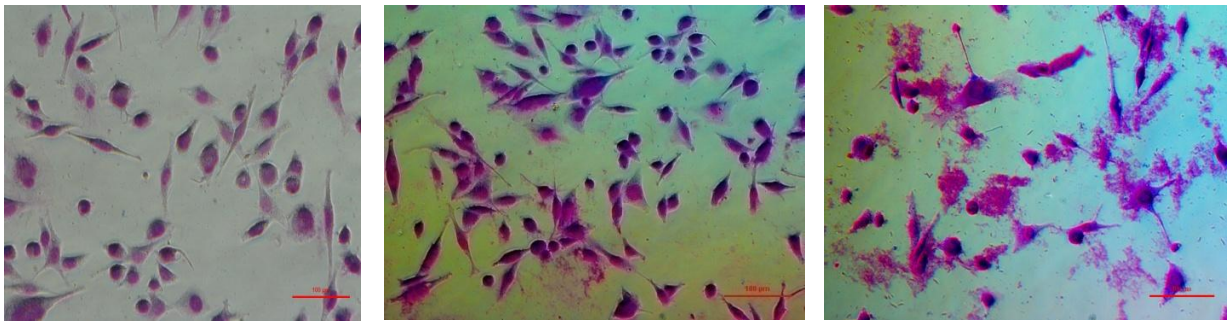
In the present study, all the lactic isolates were analyzed for their tolerance to bile up to a concentration of 1%. All the lactic cultures were able to survive in 1% concentration of bile salts with a simultaneous production of bile salt hydrolase as indicated by qualitative assay and confirmed by detection of *bsh* gene in all the isolates.

Table 2, presents the percent cellular auto aggregation of the isolates ranged from 19.0-35.0 with the maximum autoaggregation in *L. paracasei* CD4 and minimum in *B. thermoruber* CD13. The hydrophobicity of isolates towards n-hexadecane ranged from 15-32% with *L. gastricus* BTM7 having the highest ability to adhere to organic solvent. The adhesion and colonization of intestinal wall by lactic cultures are desirable properties of probiotic LAB which are dependent on the surface properties of the strain. The results in the present study gave an insight into the role of hydrophobic interactions in adhesion, followed by adherence to Caco2 cell lines.The microscopic observations of adherence of the strain to Caco 2 cells revealed that adhesion of strains CD4 and BTM7 was comparable to that adhesion of the standard strain. The adherence ability was recorded as the number of cells in each microscopic field, and the number of bacteria attached to each of those cells.Aggregation percentage was significantly higher in

isolate *L. paracasei* CD4 and *L. gastricus* BTM7 as compared to other strains, and statistically comparable to the reference strain of *L. rhamnosus* GG.

Table 2: Characterization of properties related to adhesion of selected probiotic isolates

LAB strains	% Autoaggregation	% Hydrophobicity	Mucin adhesion(%)
***L. paracasei* CD4**	35^{ef}±1.4	31^{cf}±2.8	53^{cd}±1.1
***L. gastricus* BTM7**	33^{ef}±0.8	32^{df}±2.3	75^{fg}±1.5
***Brevi. aydinogluensis* BTM9**	26^{de}±1.8	23^{ac}±1.8	34^{a}±2.0
***Brevi. thermoruber* CD13**	19^{bd}±1.2	25^{bd}±1.2	54^{cd}±1.5
***Enterococcus sp.*GTM14**	31^{ef}±2.1	15^{a}±0.9	37^{a}±2.0
***Brevi. thermoruber* HM29**	31^{ef}±1.7	25^{bd}±1.4	42^{ab}±2.5
***Brevi. thermoruber* HM34**	26^{ce}±1.3	28^{be}±1.1	40^{a}±1.5
***W. confuse* CD1**	29^{de}±2.5	21^{ab}±1.7	52^{d}±1.5
***L. fermentum* K75**	12^{ab}±1.9	37^{fg}±2.5	62^{de}±2.5
***L. fermentum* K78**	12^{ab}±1.6	34^{eg}±2.0	68^{ef}±1.1
***L. plantarum* K84**	^{14ab}±2.0	42^{g}±1.0	35^{a}±0.5
***L. plantarum* K90**	16^{bc}±1.6	30^{cf}±2.0	52^{d}±2.0
***P. acidilactici* K94**	12^{ab}±0.9	30^{cf}±1.5	41^{a}±1.5
***P. acidilactici* K98**	41^{f}±2.3	20^{ab}±1.0	70^{eg}±0.5
***L. fermentum* K100**	5^{a}±0.2	37^{fg}±2.0	51^{bc}±3.2
***L. rhamunosus* GG**	31^{e}±1.8	42^{g}±1.5	78^{g}±2.0



A) B) C)

Figure 1: Adhesion of probiotic isolates to caco2 cell lines. A) Negative control B) *L. rhamnosus* GG C) *L. paracasei* CD4

The percentage activity for all the isolates was in the range 63% to 69% for DPPH and 87% to 93% for ABTS. Both the assays revealed that *L. fermentum* K75 possessed the highest anti-

oxidant activity. The free radical scavenging activity for *L. paracasei* CD4 and *L. gastricus* strain BTM7 was above 50% whereas rest of the isolates exhibited activities around 35%. The cell free extracts of LAB have already been reported for their antioxidant activities before [29, 30].

All the isolates were positive for bile salt hydrolase activity as depicted by white precipitation around the colonies on bile supplemented agar plates as well as by PCR based amplification. As indicated by presence of BSH activity, the cultures may be useful in controlling serum cholesterol levels either by cholesterol incorporation in the cell wall, adhesion to cell wall or enzymatic reduction via cholesterol reductase. The results indicated that the *L. plantarum* K90 possessed the highest cholesterol lowering activity ($p<0.05$). Mucoid colonies were observed in all the isolates in MRS broth supplemented with sucrose after three days of incubation confirming EPS production by LAB. The EPS produced by LAB provides high viscosity, consistency and texture to the fermented product.

Conclusion

A total of 15 Isolates were able to fulfill the criteria of these isolates for being a probiotic as per DBT and ICMR guidelines. Varying degree of antimicrobial activity against *C. sakazakii* was observed for the isolates. The role of organic acids as key component possessing antimicrobial activity was confirmed. The isolates possessed biofilm inhibitory activity against *C. sakazakii.*

Objective 3: To study the effect of selected isolates for probiotic properties using model system *Caenorhabditis elegans*

Probiotic LAB are reported to regulate host intestinal health by regulating intestinal microflora directly or indirectly. The *in vitro* methods are generally suggested to screen the potential probiotic candidate, however, to further increase selection efficiency of a probiotic candidate, *C. elegans*, a free living bacterivorous soil nematode, has been suggested as an *in vivo* model. This *in vivo* model is one step closer to a multicellular animal system with intact intestinal microbiota [31]. Additionally, the studies in the nematode offer a simple, rapid, and economic screening of potential probiotic bacteria [32]. Therefore, *C. elegans* has been successfully used as established model to study antioxidative, anti-infective and lifespan extending impact of lactobacilli. We used a combination of *in vitro* and *in vivo* approaches, including Caco-2 cell cultures and the *C. elegans* nematode model, to select specific strains displaying beneficial host-microbe interactions..

Methodology:

The wild type strain of *C. elegans* (Bristol N2) (from Caenorhabditis Genetics Center, University of Minnesota, USA) was used in the study. The worms were maintained routinely using standard protocol on Nematode growth medium (NGM) agar plates seeded with *E. coli* OP50 (Uracil auxotroph, conventional food source for *C. elegans*) at 20°C. The age synchronized L4 nematode for the assay was obtained using the gravid nematodes which were bleached with solution containing house hold bleach and 5M potassium hydroxide (1:1) and were maintained in freshly seeded NGM plates for 2 days.

Liquid killing assay was performed with countable number of hermaphrodite young adult worms (~10) was transferred from NGM plates seeded with the lawn of *E. coli* OP50. The worms were washed thrice in sterilized M9 buffer (3 g/L $KHPO_4$, 5 g/L NaCl, 6 g/L Na_2HPO_4,1 ml 1 M $MgSO_4$)[17]. The entire experiment was done in 24 well plate in a liquid medium containing M9 buffer with overnight grown lactic acid bacteria of OD_{600nm} = 0.5 and *E. coli* OP50 was taken as a control. The plates were incubated at 20°C till the end of the experiment (7-10 days) to check for the change in physiological, morphological, survival and longevity of *C. elegans* fed with

LAB. Parent worms were periodically moved to a new well after every two days to separate them from the progenies. The worms were considered to be dead when it was not able to show any movement or any response to external stimuli (gentle touch with a platinum loop).

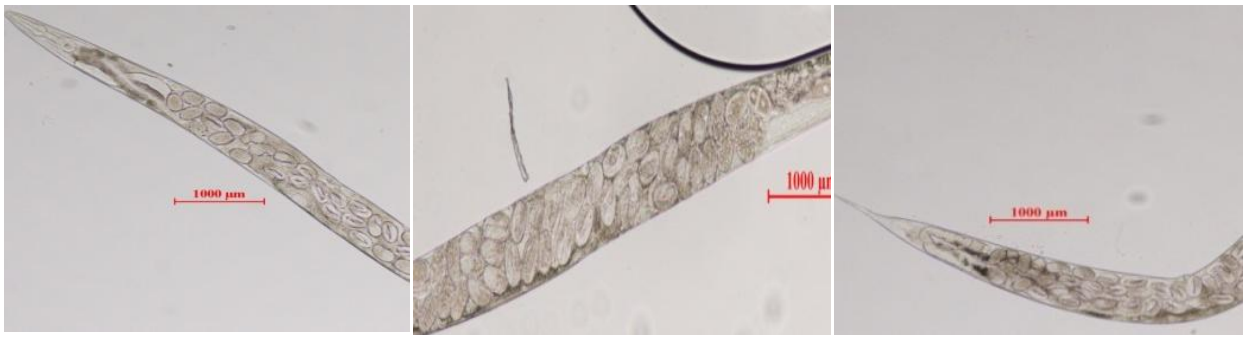
Binary Choice assay of *C. elegans* was performed to examine the preference of worms towards different probiotic bacteria; binary choice assay was conducted taking *E. coli* OP50 as preferred food [33]. Pharyngeal pumping assay was done to assess the grinding movement of the pharynx. For that the L4-stage worms in NGM plates seeded with lactic acid bacteria were incubated at 20°C and pharyngeal pumps were counted after every 24 h for 7 days. The pumping rate was determined by counting the number of contractions in the terminal bulb of the pharynx for 10 consecutive seconds.

Intestinal colonization of LAB in *C. elegans* was measured by incubating approximately (n=25) worms in wells containing LAB ($OD_{600\ nm}$ =0.5). Pretreatment with LAB isolates were given in different intervals (24 h, 48 h and 72 h) in a sterile 24 well plate (Thermo fisher scientific, New York, USA) (Non-adherent). After pretreatment, worms (n=25) were thoroughly washed with M9 buffer for the removal of excess bacteria attached to the surface of the nematodes. A total of 10 nematodes were transferred to the 1.5 ml micro centrifuge tube with the final volume of 200 µL with sterile M9 buffer. The worms were crushed using a worm crusher (micro pestle). The suspension was serially diluted and plated on MRS agar plates using spread plate method and incubated at 37°C for 24 h to get the CFU count of respective isolates [34]. The *in vivo* colonization of LAB inside *C. elegans* was examined in LAB pretreated *C. elegans* using fluorescent microscopy.

Results and Discussion:

The results showed that the LAB has increased the lifespan of *C. elegans.* The cultures *L. paracasei* CD4, showed maximum increase in survival of the worm as compared to control *E. coli* OP50 with a minimum survival with *P. acidilactici* K98 (p<0.05). A 40% of survival of the worms was seen in standard strain *L. rhamunosus* GG on the 7th day of incubation. The complete killing of nematode in control was observed in 10 days, however, *L. paracasei* CD4, *L. gastricus* BTM7, *L. plantarum* K90 enhanced the lifespan by 5 days along with standard strain *L.*

rhamunosus GG. Among the other physiological and morphological changes, all the worms possessed normal growth, well developed intestine and eggs, with normal reproduction



A) B) C)

Figure 2: Stereomicroscopic images of physiological and morphological changes in C. elegans fed with different lactic acid bacteria. A) *Lactobacillus paracasei* CD4 B) *Lactobacillus rhamunosus* GG C) *E. coli* OP50 (control)

Binary choice assay was carried out to confirm the lifespan extension effect of cultures. The results indicated that worms exhibit preferences for specific probiotic cultures (Fig. 2), suggesting that the lifespan extension effect of lactic cultures was not due to dietary restrictions. The results showed that during the first hour the number of worms in both the zones was found to be approximately equal. Around 80-90% of worms were found to use *L. paracasei* CD4, *L. gastricus* BTM7, *Brev. thermoruber* HM29, and *L. plantarum* K90 as foods indicating the attraction of *C. elegans* more towards LAB as compared towards zone B with *E. coli* OP50 where the count was less.

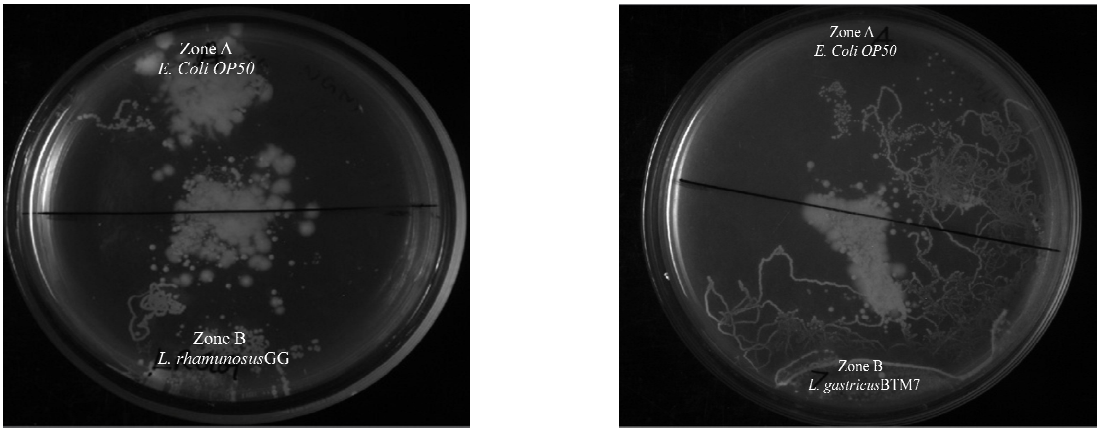


Figure 3: Binary Choice assay for food preference of C.elegans towards probiotic cultures, E. coli OP50 marked as zone A and LAB as zone B. The worms were allowed to move freely on the agar plate for 24 h to track the path of C. elegans.

The results indicated that the tested LAB did not possess any significant impact on pharyngeal pumping rate of *C. elegans* (results not shown). The pharynx was intact and rate of pharyngeal pumping was approximately similar to that of control *E. coli* OP50 even after 7 days of incubation. Although the frequency of pharyngeal pumping generally declined with age, no significant changes in the pumping rate was observed in the worms fed with probiotic cultures than in *E. coli* OP50-fed worms on day 10.

All the probiotic cultures were assessed for their ability to colonize the intestine of *C. elegans* and the colonization potential was scored by CFU count assay (Table 3). The results indicated the higher bacterial colonization with increase in incubation time up to 72 h. The colonization potential varies between Log_{10} 4 to 6 CFU per worm. The results indicated that colonization ability is strain-specific characteristic as different lactobacilli possess different colonization ability. This distinct behavior could be due to either an initial greater persistence of a lactic culture or preference of a culture to get establish in precise sections of the gastrointestinal tract of the worm.

Table 3. Density of probiotic cultures (Log_{10} CFU per worm) in the *C. elegans* intestine during 72 h of incubation

Isolates	24 h	48h	72h
Lactobacillus paracasei CD4	$5.25^{cd,x}$	$5.30^{b,x}$	$5.5^{de,x}$
Lactobacillus gastricus BTM7	$4.00^{ab,x}$	$4.30^{a,x}$	$5.00^{ab,y}$
Brevibacillus aydinogluensis BTM9	$5.27^{cd,x}$	$5.36^{b,x}$	$5.53^{de,x}$
Brevibacillus thermoruber CD13	$5.60^{d,x}$	$6.00^{c,x}$	$6.00^{f,x}$
*Enterococcus sp.*GTM14	$5.23^{cd,x}$	$5.30^{b,x}$	$5.41^{de,x}$
Brevibacillus thermoruber HM29	$4.60^{b,x}$	$5.04^{b,y}$	$5.20^{bcd,z}$
Brevibacillus thermoruber HM34	$4.84^{c,x}$	$5.00^{b,x}$	$5.11^{bc,x}$
Weissella confuse CD1	$4.47^{ab,x}$	$5.07^{bc,y}$	$5.41^{de,y}$
Lactobacillus rhamunosus GG	$4.30^{ab,x}$	$5.00^{b,y}$	$5.07^{ab,y}$
Lactobacillus fermentum K75	$5.07^{cd,x}$	$5.40^{b,y}$	$5.55^{de,y}$
Lactobacillus fermentum K78	$5.00^{cd,x}$	$4.47^{b,y}$	$5.00^{ab,y}$
Lactobacillus plantarum K84	$5.11^{cd,x}$	$5.17^{b,x}$	$5.25^{bc,x}$
Lactobacillus plantarum K90	$5.20^{cd,x}$	$5.40^{b,x}$	$5.60^{e,x}$
Pediococci acidilactici K94	$5.30^{cd,y}$	$4.69^{a,x}$	$5.07^{ab,xy}$

Pediococci acidilactici K98	$5.0^{cd,x}$	$5.17^{b,x}$	$5.46^{cde,x}$

a-f Means in the column with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. ($p<0.05$)

x-z = Means in the row with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications.

Conclusion:

Strain specific response in *C. elegans* fed with probiotic was observed whereby all the isolates increased the life span of the worms without any alterations in the morphology and physiological responses. Higher preference towards probiotic isolates for food was also observed.The isolates were able to colonize the gut of the worm after 24 h of incubation.Out of 15 isolates two isolates i.e. *Lactobacillus gastricus* BTM7 and *Lactobacillus plantarum* K90 showed promising results and were used for further analysis.

Objective 4: To study the protective effect of probiotics against infection with *Cronobacter sakazakii* in *Caenorhabditis elegans*

C. sakazakii is an opportunistic pathogen and an occasional contaminant in powdered infant formula, as infant food made of powdered infant formula products may be a source and vehicle of infections causing meningitis, bacteremia and necrotizing enterocolitis in neonates with case fatality rates ranging between 40% and 80% [18, 19]. The antibacterial activity of LAB is mainly attributed to their ability to produce various inhibitory compounds such as diacetyl, organic acids, hydrogen peroxide (H_2O_2) and bacteriocins during lactic fermentations. Although probiotic cultures have been reported for their antimicrobial activities against an array of pathogens leading the gastrointestinal disorders, however very few studies have been conducted to check the efficacy of probiotics against *C. sakazakii.* Therefore, this study was designed to identify the probiotic LAB cultures for their antimicrobial activities against *C.sakazakii*. Further, the selected strains were evaluated for their effectiveness in excluding the colonization of *C.elegans* by *C.sakazakii*.

Methodology

Antimicrobial activities of selected LAB against *C.sakazakii*

CFS of probiotic bacteria isolated from fermented dairy products and fermented foods was prepared. Antimicrobial susceptibility of probiotics against *C. sakazakii* was measured using agar well diffusion method [35].MIC was determined as the lowest concentration that produces visible disruption in biofilm formation and significant reduction in the readings when compared with the control wells at $OD_{570\ nm}$ [36]. The effect of CFS on the inhibition of *C. sakazakii* was determined by quantifying the biofilm biomass through microtitre plate (MTP) assay [37]. The effect of CFS on the biofilm inhibition of *C. sakazakii* was determined by quantifying the biofilm biomass through microtitre plate (MTP) assay. Post biofilm inhibition by CFS of probiotic isolateswas done by the method given by [38].

Pathogenic potential of *C. sakazakii* in *C. elegans*

Liquid killing assay of *C. elegans* infected with *C. sakazakii* was done by the method given by[39]. Physiology and morphology of worms infected with *C. sakazakii* was observed under stereo microscope (Nikon SMZ1000, Japan). Chemotaxis and pharyngeal pumping assay was done by the method employed by [21]. Preparation of worm (Probiotic treated) total RNA and RT-PCR was done by method given by [40].

Results and Discussion:

The results of antimicrobial activities of fifteen probiotic isolates against *C. sakazakii* as determined by the different fractions of cell free supernatant of lactic cultures are presented in Table 4.

Table 4. *In vitro* antimicrobial activity of probiotic bacteria against *C. sakazakii* (ATCC 12868)

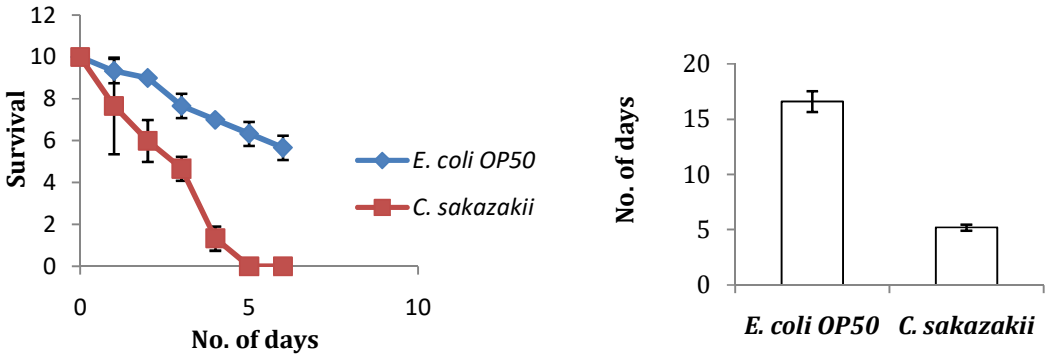
Isolates	Diameter of zone of inhibition in mm		
	Cell Free Supernatant (CFS)	Heat neutralized cell free supernatant (hCFS)	pH Neutralized Cell Free Supernatant (pH 6.8) (nCFS)
L. paracasei CD4	20^{bd}±1.1	20^{ce}±1.1	-
L. gastricus BTM7	24^{e}±1.1	22^{e}±1.1	-
Brev.. aydinogluensis BTM9	18^{ac}±0	18^{be}±1.1	-
Brev. thermoruber CD13	18^{bd}±1.1	16^{bc}±1.1	-
*Enterococcus sp.*GTM14	18^{bd}±1.1	18^{be}±1.1	-

Brevi. thermoruber HM29	18^{a}±1.1	20^{de}±0	-
Brevi. thermoruber HM34	20^{bd}±1.1	18^{be}±1.1	-
W. confuse CD1	16^{ab}±1.1	16^{bc}±1.1	-
L. rhamunosus GG	20ce±1.1	18^{be}±1.1	-
L. fermentum K75	16^{a}±1.1	16^{ab}±0	-
L. fermentum K78	18^{ab}±1.1	20^{ce}±1.1	-
L. plantarum K84	20^{bd}±1.1	18^{bd}±1.1	-
L. plantarum K90	22^{de}±0	22^{e}±1.1	-
Pedio. acidilactici K94	18^{bd}±1.1	18^{be}±1.1	-
Pedio. acidilactici K98	20^{bd}±1.1	18^{bd}±0	-
L. fermentum K100	16^{a}±1.1	14^{a}±1.1	-

a-e Means in the column with different superscript letter are significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. ($p<0.05$)

In our present study we have found that the cell free supernatant of probiotic bacteria was able to inhibit the biofilm formed by food pathogen *C. sakazakii*. The effects of CFS of selected probiotic strains on the biofilm formation and disruption of mature biofilms by *C. sakazakii* were analyzed by using standard crystal violet assay. The co-incubation experiment where the cultures were tested for their ability to inhibit the biofilm formation using all fractions of CFS (CFS, hCFS and nCFS) indicated that the untreated and heat treated CFS had maximum inhibitory activity against formation of biofilm whereas the neutralized supernatant was the weakest among all tested fractions. The maximum inhibition in wells treated with untreated CFS of *L. paracasei* CD4 and *L. gastricus* BTM7 and the lowest inhibition was observed for *L. fermentum* K75 (48%). Further the ability of the probiotic strains were tested to eradicate the mature biofilm of *C. sakazakii* using post incubation assay, where the biofilm formed after 12, 24 and 48 h was treated with different fractions of CFS (treated and untreated) and results were expressed in % biofilm inhibition potential. Similar to co-incubation assay, the untreated CFS resulted in maximum biofilm eradication ability followed by hCFS and minimum inhibitory activity with pH neutralized CFS.

The pathogenic potential of *C. sakazakii* was determined by feeding *C. elegans* with *C. sakazakii* to check the survivability of the worms.



a. Survival b. Mean Life Span

Figure 4(a): Survival curve showing survival and mean life span of *C. elegans* fed with *C. sakazakii*.

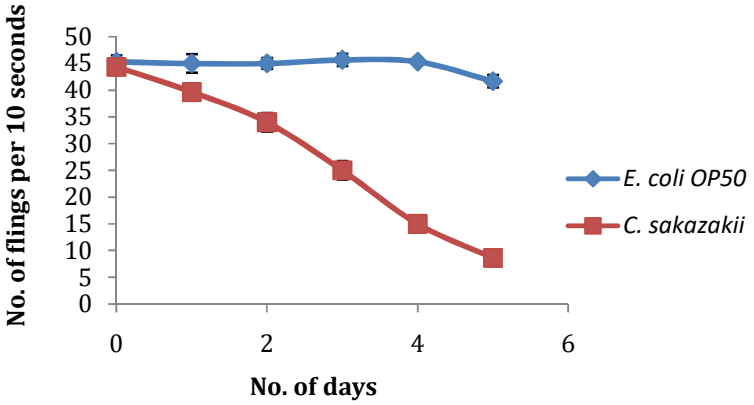


Figure 4(b): Pharyngeal pumping rate of the terminal bulb of pharynx in control *E. coli* OP50 and in *C. sakazakii* infected worm recorded as the number of flings in 10 consecutive seconds for six days.

Protective role of probiotic cultures against *C. sakazakii* infection was observed in three different competitive exclusion assays:

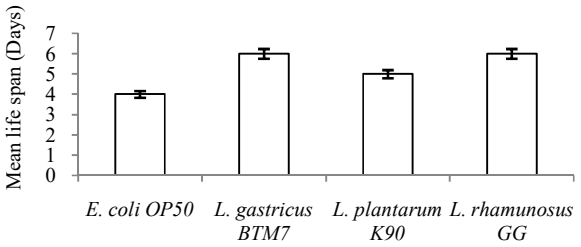


Figure 5(a): Competition assay

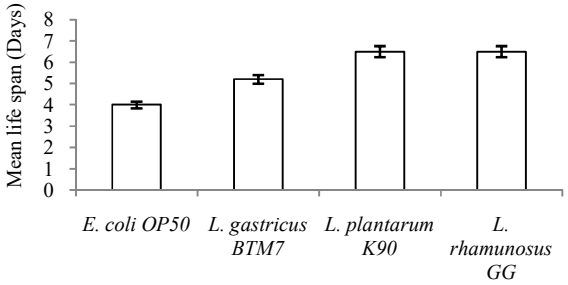


Figure 5(b): Displacement assay

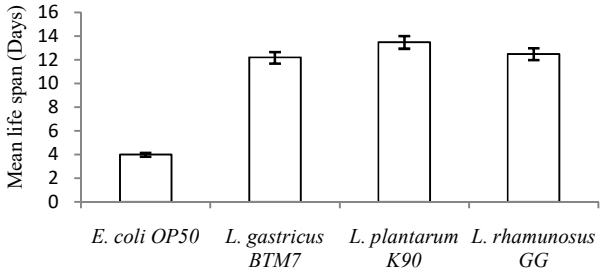


Figure 5(c): Exclusion

Conclusion:

The traditional fermented foods are a reservoir for excellent candidates for probiotics. All the cultures present in these sources do not possess the probiotic attributes. Feeding with probiotic lactic cultures is effective to the extend lifespan in *C. elegans* and the colonization ability differs among the strains. Pre-treatment of worms with probiotic isolates eradicates the colonization by

C. sakazakii in C. elegans.The isolated strains could be used as pro-phylactic measures to prevent infections with *C. sakazakii*

Overall conclusion:

The strains from dairy products and fermented cereal products could be excellent candidates for probiotics. All the cultures present in these sources do not possess the probiotic attributes. Feeding with probiotic lactic cultures is effective to the extend lifespan in *C. elegans* and the colonization ability differs among the strains. Pre-treatment of worms with probiotic isolates eradicates the colonization by *C. sakazakii in C. elegans*. The isolated strains could be easily used as pro-phylactic measures to prevent infections with *C. sakazakii.*

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Publications

1. **Kavita Sharma**, Rishi Mahajan, Sampan Attri and Gunjan Goel (2017). Selection of indigenous *Lactobacillus paracasei* CD4 and *Lactobacillus gastricus* BTM 7 as probiotic: assessment of traits combined with principal component analysis. *Journal of Applied Microbiology* 122(5): 1310-1320.**(I.F: 2.16, Scopus indexed)**
2. **Kavita Sharma**, PooranachithraMurugesan, Krishnaswamy Balamurugan and Gunjan Goel (2018). Multivariate Analysis of Increase in Life Span of *Caenorhabditis elegans* Through Intestinal Colonization by Indigenous Probiotic Strains. *Probiotics and Antimicrobial Proteins.*1-9.**(I.F: 2.34, Scopus indexed)**

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4. Megha Bhatnagar, Sampan Attri,**KavitaSharma,** Gunjan Goel (2017). *Lactobacillus paracasei* CD4 as potential indigenous lactic cultures with antioxidative and ACE inhibitory activity in soymilk hydrolysate. *Journal of Food Measurement and Characterization*12 (2), 1005-1010 **(I.F: 1.181, Scopus indexed)**
5. Sampan Attri, **KavitaSharma**, Pinky Raigond and Gunjan Goel (2018). Colonic fermentation of polyphenolics from Sea buckthorn (*Hippophae rhamnoides*) berries: Assessment of effects on microbial diversity by Principal Component Analysis. *Food Research International*, 105: 324-332.**(I.F: 3.52, Scopus indexed)**
6. Rishi Mahajan, Sampan Attri, **KavitaSharma,**Niharika Singh, Deepika Sharma and Gunjan Goel (2018). Statistical assessment of DNA extraction methodology for culture-independent analysis of microbial community associated with diverse environmental samples. *Molecular Biology Reports.* 45(3),297-308**(I.F: 1.889, Scopus indexed)**
7. Shagun Choudhary, Manisha Singh, Deepak Sharma, Sampan Attri, **KavitaSharma** and Gunjan Goel (2018). Principal Component Analysis of Stimulatory Effect of Synbiotic Combination of Indigenous Probiotic and Inulin on Antioxidant Activity of Soymilk. *Probiotics and antimicrobial proteins*. pp.1-7.**(I.F: 2.34, Scopus indexed)**

Book Chapter

1. Niharika Singh, Sampan Attri, **Kavita Sharma** and Gunjan Goel (2016). Indigenous Fermented Foods and Beverages. In Chetan Sharma, Anil K. Sharma, and K.R. Aneja,*Frontiers in Food Biotechnology*(pp. 47-74). USA: Nova Science Publishers. [ISBN: 978-1-63484-679-0]**(Book chapter, Scopus indexed).**

Conference posters

1. **Kavita Sharma,** Mamta Raghav and Gunjan Goel(2015). Identification of Potential Lactic Acid Bacteria from Indigenous Fermented Foods of Himachal Pradesh for Probiotic Attributes. *Proceedings of the Bioprocessing India,* [IIT Madras, Chennai : 17-19 Dec, 2015].
2. **Kavita Sharma**, Gunjan Goel. Biodiversity of Lactic Acid Bacteria in Himachali fermented food (Babroo) and its screening for various probiotic attributes. The International Conference held during 6th, 7th and 8th October, 2016 on *Biodiversity: Current scenario and future strategies* (Organized and hosted St.Bede's College Shimla).
3. **Kavita Sharma**, Gunjan Goel. Functional atttribues of potential probiotic *Lactobacillus paracasei* (CD4) and *Lactobacillus gastricus* (BTM 7) isolated from dairy products. *International conference on Recent Trends in Microbiology (RTM-2016) (*Organized and hosted by Alagappa University, Karaikudi, Tamil Nadu*).*
4. Sampan Attri, **Kavita Sharma**, Gunjan Goel (2017). Effect of simulated gastric and small intestine digestion on microstructure of seabuckthorn berries extract. *Proceedings of the Advances in Food Science and Technology* [Eternal University, Baru Sahib, H.P. : 24-25 March, 2017], pp.-Google Citation
5. Surabhi Chauhan, **Kavita Sharma**, Gunjan Goel (2017). Antimicrobial Activity of Cell Free Supernatant of Indigenous Lactic Acid Bacteria against Cronobacter

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6. Shagun Choudhary, Manish Singh, **Kavita Sharma**, Sampan Attri, Deepak Sharma, Gunjan Goel (2017). Effect of prebiotics on growth of indigenous lactic culures in fermented soymilk. *2nd Himachal Pradesh Science Congress on Science and technology for sustainable livelihood in Indian Himalayan Region*held from (November 20-21, 2017) at Hotel Peterhoff, Shimla.

Workshops

1. Attended two week GIAN (Global Initiative of Academic Networks) course on ***"Functional Foods and Nutraceuticles: Fundamental and Mechanistic Approaches"*** held on 11th December to 22nd December 2017 at Central university of Haryana, Mahendergarh (Sponsored by Ministry of Human Resource Development (MHRD) under the scheme Global Initiative for Academic Network).
2. Attended workshop on ***"Patent Drafting"*** held on 13th October 2017 *at Jaypee* University of Information Technology, Waknaghat, Solan, Himachal Pradesh.
3. Attended DBT sponsored national seminar cum workshop on ***"Genomics and Proteomics-2017*** (February 8-10, 2017) at Alagappa University, Science Campus, Karaikudi, Tamilnadu.
4. Attended workshop on ***"Innovation and Intellectual Property Rights"***held on 9th December 2016 at Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh.